Nanocarrier based small interfering RNA (siRNA) system for targeting ocular neovascularization

Summary for the degree

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1. Introduction

Vision impairment due to the progression of various disease conditions is a major health problem and is increasing daily. World health organization (WHO) recently reported that among the total world population, 457 million adults are suffering from diabetes of which 285 million patients are visually impaired because of ocular neovascularization (NV) [1]. Major diseases that contribute to visual impairment are age-related macular degeneration (AMD), diabetic retinopathy (DR), retinopathy of prematurity (ROP), retinoblastoma, choroidal neovascularization (CNV), retinal vein occlusions, etc. [2]. A survey study has projected that in coming years the cases of vision loss due to AMD might be 196 million which if not controlled may reach to 288 million in 2040, with maximum cases from Asia[3]. Angiogenesis plays an important key role in vasculature formation which is responsible for the growth and metastasis of hematogenic factors[4]. It involves in the fundamental process of the formation of new vessels from pre-existing vessels. However, uncontrolled growth of new vessels leads to neovascularization which may lead to loss of vision if not controlled in time[5]. This uncontrolled vessel formation spreads from the retina to the vitreous cavity leading to hemorrhage and destruction of the retina ultimately leading to damage to corneal sites[6]. Neovascularization contributes to the progression of various diseases. Factors like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factors (PDGFs), etc. [7] govern the process of angiogenesis in the cells[8]. Among these VEGF is more prominent. Many clinical studies have proved the major role of VEGF in the regulation of angiogenesis in the body[9]. VEGF signaling is responsible for the pathological progression and development of angiogenesis. In retinal

cells, when the process of angiogenesis goes out of control it leads to overexpression of VEGF. Overexpression of VEGF leads to the formation of vasculature in the ocular sites which may lead to blindness. Various drugs have been tested to act as potential anti-angiogenic agents by targeting VEGF- pathway[10].

VEGF AND OCULAR NEOVASCULARIZATION

In the eye, various studies have confirmed the decisive role of VEGF in retinal vasculature growth and development. Retinal epithelial and muller cells are the foremost sources of VEGF and they show their effects through various receptors which are generally present in endothelial cells, microphages as well as monocytes [11]. Signaling of VEGF also maintained the corneal transparency and avascular state. There are various members of the VEGF family among which VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E are known to date[12]. VEGF is particularly known as VEGF-A, while others are virally encoded and involved in the process of lymphogenesis. VEGF-A has various isoforms VEGF121, 165, 189,206, etc. of which VEGF121 and VEGF165 are most profoundly found in humans[13]. VEGF signaling is regulated by VEGFR-1, VEGFR-2, and VEGFR-3 of which 1 and 2 are involved in the angiogenesis and tissue vascularization process[14]. Various studies have demonstrated that intravenous administration of VEGF in animals causes retinal vascularization which plays a key role in the development of various ocular diseases. In the overexpression of VEGF, various VEGF mRNA plays an important role[15]. Messenger RNA (mRNA) is well known for its role in the synthesis of particular proteins which regulates specific gene expression. Targeting the synthesis of this mRNA could be a better approach to preventing and controlling ocular neovascularization.

2. Current status of research & development

Several methods have been used traditionally for the treatment of ocular neovascularization which includes topical administration of corticosteroid and nonsteroidal anti-inflammatory drugs, mechanical treatments like laser photocoagulation, photodynamic therapy, photodynamic therapy, macular translocation, and surgical removal[16]. However, corticosteroids lead to superinfection, glaucoma, and cataract in many cases. The main drawback with mechanical therapies is that they lead to a lack of visual improvement, NV recurrence, and damage to surrounding retinal tissue and that's why not commonly applied in clinical practice[17]. More recently, monoclonal anti-VEGF antibodies like bevacizumab, pegatinib (Avastin®), ranibizumab (Lucentis®), and pegaptanib (Macugen®), etc. are used as the gold standard for the treatment of ocular neovascularization. Nevertheless, prolonged use of these antibodies leads to endophthalmitis and retinal detachment [18].

Hence, it is very important to target the molecular mechanism involved in the pathogenesis of NV rather than targeting external factors. RNAi therapy works on the same principle, siRNA targeting particular expression of mRNA responsible for the expression of the targeting gene is targeted. Inhibiting the expression of genes involved in the progression of the disease could be a great approach to the management and control of disease prevalence.

3. Conceptual framework and rationale of the study

Ocular NV plays a key role in the pathogenesis of many ocular diseases, which ultimately leads to loss of vision. Several growth factors are involved in the progression of ocular NV. Targeting the most prominent growth factor involved in neovascularization could be a superior molecular target for controlling ocular NV[19]. Recently, RNAi therapy is getting

the attention of many formulation scientists because of its highbrow mechanism of aiming genes involved in the pathogenesis of diseases[20]. RNA interference (RNAi) is a central process that regulates the expression of various genes in the cells[21]. The key principle involved in RNAi therapy is that it acts on the misleading complementary base pairing of targeted mRNA thereby leading to the degradation of mRNA transcripts by cellular RNAse [22]. This therapy acts straightway on disease-causing genes thereby lowering the toxicity and other side effects. Hence, siRNA targeting VEGF expression could be a promising approach for the treatment of ocular neovascularization[23]. VEGF expression was regulated by various proteins synthesized by the process of translation in the cells. siRNA targeting complementary mRNA responsible for the generation of proteins regulating VEGF expression could be a new therapeutic aspect for controlling ocular neovascularization.

Rationale of the study:

VEGF plays a crucial role in retinal vasculature growth and development. VEGF-A is a key growth factor involved in neovascularization and angiogenesis. Its expression is regulated by HIF-α in response to hypoxia. HuR stabilizes VEGF-A mRNA post-transcriptionally. Targeting HuR with siRNA can effectively lower VEGF levels and potentially prevent conditions like diabetic retinopathy. However, delivering siRNA to the eye is challenging. Cationic polymers and lipids can be used as carriers to protect siRNA and facilitate its targeted delivery. A lipid/polymeric-based siRNA delivery system could provide a controlled release and long-term treatment for ocular neovascularization. Encapsulating HuR-siRNA in lipoplexes has shown promise in reducing HuR expression and VEGF protein levels.

4. OBJECTIVES

Objectives of the work include are as follows:

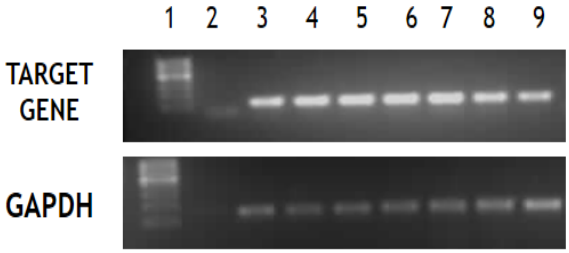
1. To develop a nano-carrier-based system for the delivery of siRNA.
2. To characterize and evaluate the designed delivery system for its physicochemical parameters and in-vitro release characteristics.
3. To study the in-vivo performance of the designed delivery system for their affinity and/or concentration at the site of interest in a suitable animal / in-vivo model.
4. Evaluating the transfection efficiency of this developed delivery system of siRNA

5. Research summary

5.1.Transfection of ARPE-19 with predesigned target gene siRNA

Method: Procured siRNA was evaluated for their efficiency in downregulating target gene mRNA. Studies were carried out using the commercial transfection agent Lipofectamine®2000 in a 6-well plate according to the manufacturer's instructions. The transfections were carried out in triplicate with i) Plain Lipofectamine ii) Lipofectamine complexed with pre-designed siRNA against the target gene iii) Lipofectamine complexed with scrambled siRNA. After four hours, the serum-free medium was replaced with Dulbecco's Modified Essential Medium complete media (without antibiotics). After 48 hours, the cells were lysed using TRIzol reagent (Invitrogen), and mRNA was isolated. The isolated mRNA was converted to cDNA using Superscript III reverse transcriptase (Invitrogen). The cDNA was analyzed by PCR using target gene primers to determine whether the mRNA of the target gene was effectively downregulated by siRNA. Amplification of cDNA was also done using GAPDH (housekeeping gene).

Results: As shown in **Figure 1** there is no downregulation of target gene mRNA by pre-designed siRNA. In further investigation, it was observed that the siRNA procured contains non-complementary nucleotides to the target gene mRNA in seed regions**.** For silencing any gene by using siRNA it must be completely complementary to the targeted mRNA sequence. Hence, there was no downregulation observed after transfection of the pre-designed siRNA sequence.



Lane 1- 100 bp DNA ladder
Lane 2- No Template Control
Lane 3- Plain cells control
Lane 4- Lipofectamine control
Lane 5,6,7- Target gene pre-designed siRNA transfected cells
Lane 8,9- Scrambled siRNA transfected cells

Figure 1: Reverse Transcriptase Polymerase Chain Ration. The cDNA obtained from mRNA of cells (treated as indicated) was amplified with target gene primes and GAPDH primers

Hence, we decided to design siRNA sequences specific for targeting the target gene.

5.2.Design of siRNA against the target gene

Method: To analyze the effect of the downregulation of genes involved in neovascularization, siRNA was designed. Various software like siDirect, siDESIGN Center, Genscript, Asi designer, BLOCK-iT RNAi Designer, RNAi Explorer, and RNAi codex have been used [24][25]. The sequences obtained from these softwares were BLASted (Basic Local Alignment Search Tool (BLAST)) using PubMed human genome and transcriptome database. The sequences showing no off-target effects were chosen and are in the process of procurement.

Results: Using the guidelines for designing siRNA, siRNA sequences were designed and analyzed on BLAST against the available human transcriptome PubMed database. The siRNA sequences were designed following guidelines given on the Thermo Fischer Scientific website (www.thermofischer .com).

Designing of primers.

Method: Primers for Reverse Transcriptase Polymerase Chain Reaction were designed and compared using the following software; Primer-BLAST, Primer3, and PerlPrimer. The primer sequences (target gene and housekeeping gene) were blasted against the human genome and transcriptome database and were confirmed to be specific against the target gene and have been procured.

Results: The primers for amplification of the target gene by polymerase chain reaction were designed by using Primer 3 software available on the National Institute of Health website (https://www.ncbi.nlm.nih.gov). Also, the off-target effects of these primers were checked by blasting them against the human transcriptome database.

5.3.Transfection of ARPE-19 with customized target gene siRNA

Method: Selected three sequences were analyzed for their efficiency in downregulating target gene mRNA by transfecting them into ARPE-19 cells. Same protocol was followed with mRNA isolation after 24, 48 hrs. post-transfection using TRIzol reagent.

Results: The PCR result (see **Figure 2**) shows that siRNA sequences 2 and 3 show better downregulation of target gene mRNA as compared to the siRNA sequence 1 in 24 and 48 hr post-transfection analysis.

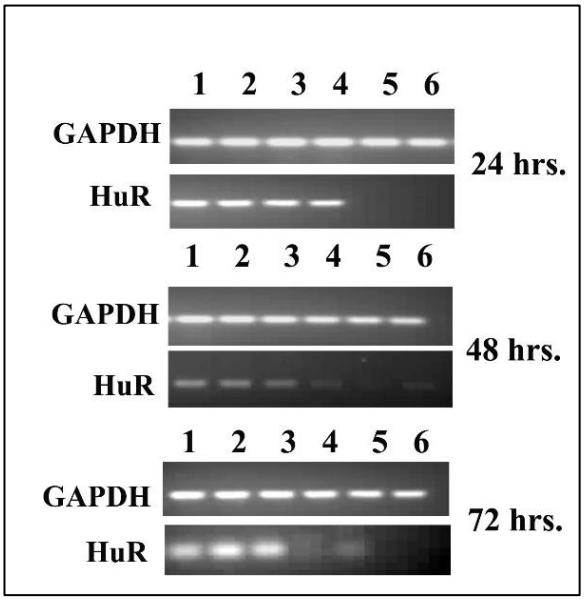


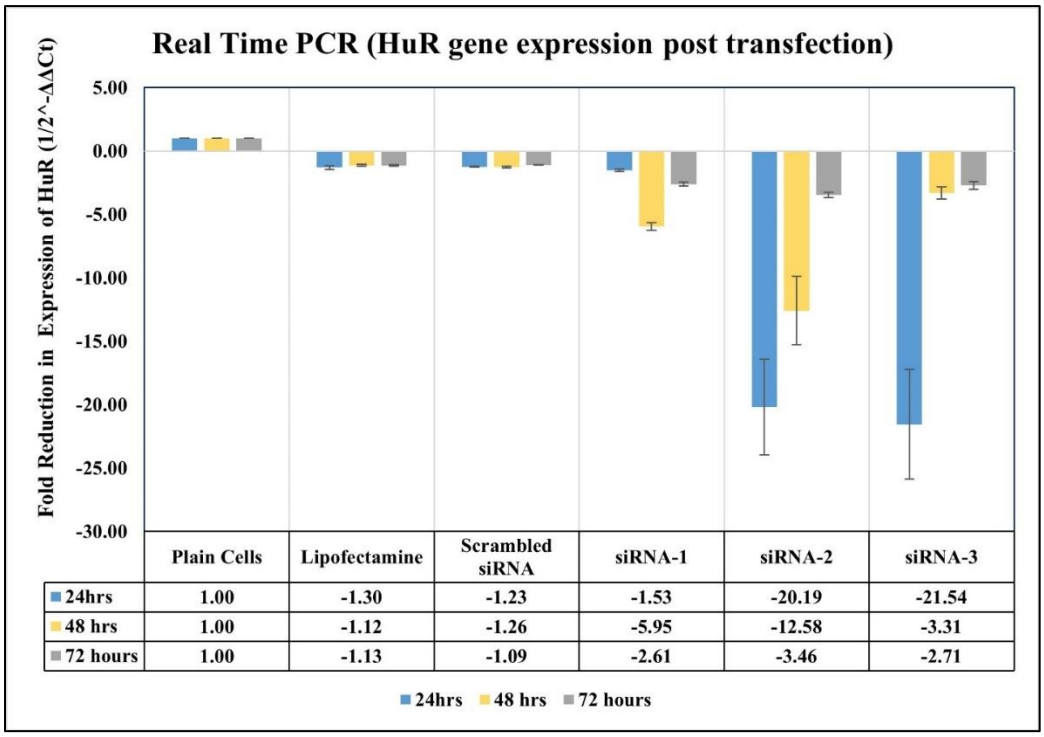
Figure 2. Reverse transcriptase polymerase chain reaction (Gel electrophoresis). All three siRNA sequences were analyzed for their efficiency in HuR downregulation. Lane sequence; Lne1- plain cells, lane 2- lipofectamine only, lane 3- scrambled siRNA, lane 4- siRNA_1, lane 5-siRNA_2, lane 6- siRNA_3

5.4.Transfection of ARPE-19 with designed target gene siRNA

Method: Procured siRNA was evaluated for their efficiency in downregulating target gene mRNA. Studies were carried out using the commercial transfection agent Lipofectamine®2000 in a 6-well plate according to the manufacturer's instructions. The transfections were carried out in triplicate with i) Control cells ii) Lipofectamine iii) Scrambled siRNA -Lipofectamine iv) siRNA_1-Lipofectamine v) siRNA_2 -Lipofectamine vi) siRNA_3-Lipofectamine. After four hours, the serum-free medium was replaced with Dulbecco's Modified Essential Medium complete media (without antibiotics). After 24,48 and 72 hours, the cells were lysed using TRIzol reagent (Invitrogen), and mRNA was isolated. The isolated mRNA was converted to cDNA using

Superscript III reverse transcriptase (Invitrogen). The cDNA was analyzed by PCR using target gene primers to determine whether the mRNA of the target gene was effectively downregulated by siRNA. RT-qPCR was performed using Power SYBR Green Mastermix (Applied Biosystems, Warrington, UK). Reactions were analyzed upon an ABI 7500 real-time PCR machine using the following cycle conditions: 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles at 57°C for 15 seconds, 72°C for 1 minute and 60°C for 1 minute. Results were normalized against GAPDH expression. The mRNA expression levels were determined according to the $2^{-\Delta\Delta Ct}$ method[26].

Results: As shown in **Figure 3** there is downregulation of target gene mRNA by all sequences on 24 hrs, 48 hours, and 72 hours post-transfection.



	Plain Cells	Lipofectamine	Scrambled siRNA	siRNA-1	siRNA-2	siRNA-3
24hrs	1.00	-1.30	-1.23	-1.53	-20.19	-21.54
48 hrs	1.00	-1.12	-1.26	-5.95	-12.58	-3.31
72 hours	1.00	-1.13	-1.09	-2.61	-3.46	-2.71

Figure 3. Gene expression study by real-time PCR. This study was performed to find out the effect of differently designed siRNA on downregulating target genes. In-vitro silencing

effect of siRNA. ARPE19 cells were treated with Lipofectamine 2000 complexed with siRNA_1, siRNA_2, and siRNA_3 respectively.

Protein isolation:

Protein isolation was done after 24, 48, and 72 hrs. post-transfection by using RIPA buffer. Retinal cells were trypsinsed using 0.05%v/v trypsin EDTA and centrifuged at 800 g for 8 mins. Then chilled PBS was added to the cell pellet followed by centrifugation at 1000g for 8 min. The supernatant was removed carefully without disturbing the pellet and 100 ul of RIPA buffer was added containing protease inhibitors. The cellular lysate was kept in an ice bath for 30 min. The lysates were then spun at 13000 x g for 10 mins. The supernatants were collected and stored in aliquots at -20°C.

Western blot

Method: SDS gel electrophoresis was performed according to standard protocols. Proteins were separated by standard SDS gel electrophoresis and then transferred onto a nitrocellulose membrane using the semi-dry transfer procedure. Membranes were blocked in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.4) containing 5% skim milk powder. Antibodies against VEGF, target gene, and β-actin were diluted in the same buffer but with 0.5% skim milk powder and incubated with the membrane for 1 h at room temperature. β-actin protein levels were measured to control equal protein loading of the wells in Western blot analysis. Membranes were washed 5-6 times with TBST. Affinity purified horseradish-conjugated swine anti-rabbit immunoglobulins were diluted 1:5000 in the blocking buffer with 0.5% skim milk powder and applied for two hours at room temperature. The bands were detected using the ECL kit (Amersham Biosciences) and quantified on a Gel Doc imaging system (Bio-Rad, India).

Results: The cellular lysates obtained at different time intervals 24 h, 48 h, and 72 h were analyzed by western blot.

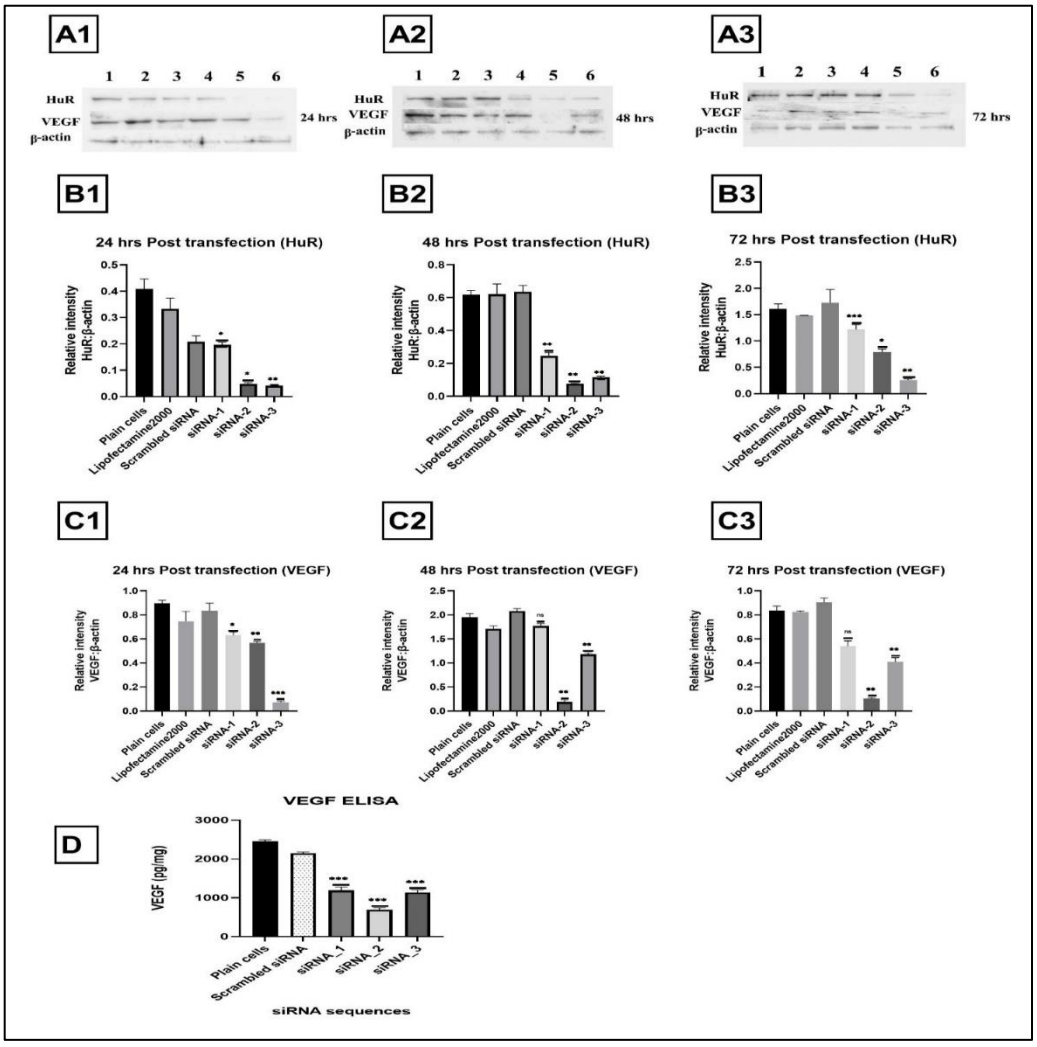


Figure 4. Western blot analysis of the cellular lysates. Lysates from the cells were loaded as follows: -Lane 1. Plain cells (untreated cells), Lane 2. Lipofectamine™ 2000 treated cells, Lane 3. Scrambled siRNA (siRNA_Sc) tr, Lane 4. siRNA_1 transfected cells, Lane 5. siRNA_2 transfected cells, Lane 6. siRNA_3 transfected cells. Both the target gene and VEGF protein were effectively downregulated by siRNA. Significant downregulation was obtained by siRNA -2 48 hours post-transfection.

5.5.Characterization of siRNA interaction studies

i. PEI as a complexing agents

Method: Two grades of PEI PEI 25kDa and 2kDA based on their cationic nature were selected for complexation with siRNA. The suitable complexing ratio of siRNA and complexing agents were calculated by using N/P ratios and analyzed on agarose gel for their complexation efficiency. Complexes of siRNA with PEI were analyzed on 2.5%w/v agarose gel for their complexation efficiency at 65 mV.

Results: Gel Retardation Assay

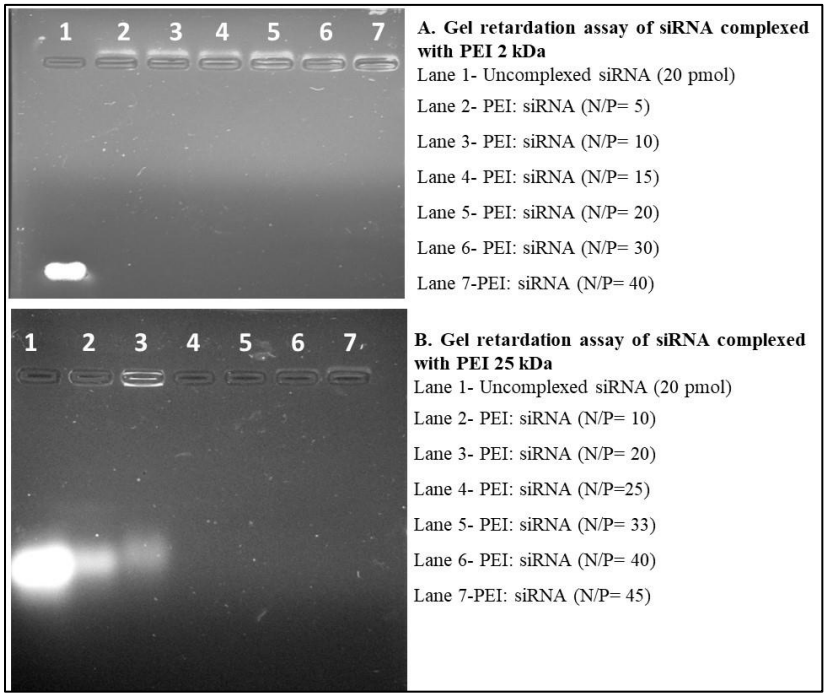

1 2 3 4 5 6 7
A. Gel retardation assay of siRNA complexed with PEI 2 kDa
Lane 1- Uncomplexed siRNA (20 pmol)
Lane 2- PEI: siRNA (N/P= 5)
Lane 3- PEI: siRNA (N/P= 10)
Lane 4- PEI: siRNA (N/P= 15)
Lane 5- PEI: siRNA (N/P= 20)
Lane 6- PEI: siRNA (N/P= 30)
Lane 7-PEI: siRNA (N/P= 40)
1 2 3 4 5 6 7
B. Gel retardation assay of siRNA complexed with PEI 25 kDa
Lane 1- Uncomplexed siRNA (20 pmol)
Lane 2- PEI: siRNA (N/P= 10)
Lane 3- PEI: siRNA (N/P= 20)
Lane 4- PEI: siRNA (N/P=25)
Lane 5- PEI: siRNA (N/P= 33)
Lane 6- PEI: siRNA (N/P= 40)
Lane 7-PEI: siRNA (N/P= 45)

Figure 5. Complexation of siRNA with PEI

A gel retardation assay was carried out to evaluate the complexation of siRNA with PEI. Higher molecular weight PEI has more transfection efficiency [27][28] and hence we have selected PEI 25kDA for the formation of lipopolyplex. The loss in the mobility of siRNA indicated that PEI

25kDa in an N/P ratio of 20:1 could condense siRNA effectively (**Figure 5A**). This ratio of PEI to siRNA was used for liposomal interaction and preparation of LPPs.

Lipopolyplex formation

Various lipid compositions were explored for the formation of liposomes. These liposomes were further incubated with PEI (25kDa-siRNA) polyplex for the generation of LPP. These siRNA complexes are further coated with lipids for *in-vitro* protection against heparin, serum, and RNase enzymes.

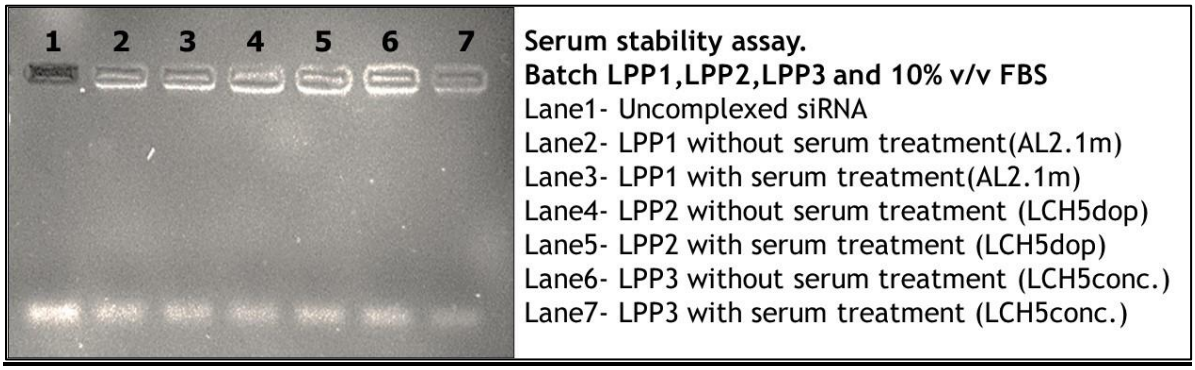

1 2 3 4 5 6 7
Serum stability assay.
Batch LPP1,LPP2,LPP3 and 10% v/v FBS
Lane1- Uncomplexed siRNA
Lane2- LPP1 without serum treatment(AL2.1m)
Lane3- LPP1 with serum treatment(AL2.1m)
Lane4- LPP2 without serum treatment (LCH5dop)
Lane5- LPP2 with serum treatment (LCH5dop)
Lane6- LPP3 without serum treatment (LCH5conc.)
Lane7- LPP3 with serum treatment (LCH5conc.)

Figure 6. Serum stability assay of all optimized lipopolyplexes. All complexes were incubated for 4hr with 10%v/v of FBS ad the siRNA was isolated by using trizol and analyzed on gel electrophoresis assay.

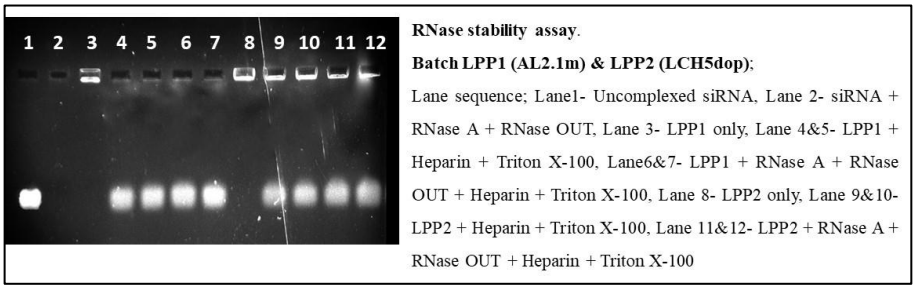

1 2 3 4 5 6 7 8 9 10 11 12
RNase stability assay.
Batch LPP1 (AL2.1m) & LPP2 (LCH5dop);
Lane sequence; Lane1- Uncomplexed siRNA, Lane 2- siRNA + RNase A + RNase OUT, Lane 3- LPP1 only, Lane 4&5- LPP1 + Heparin + Triton X-100, Lane6&7- LPP1 + RNase A + RNase OUT + Heparin + Triton X-100, Lane 8- LPP2 only, Lane 9&10- LPP2 + Heparin + Triton X-100, Lane 11&12- LPP2 + RNase A + RNase OUT + Heparin + Triton X-100

Figure 7. RNase stability assay for LPP1 and LPP2.

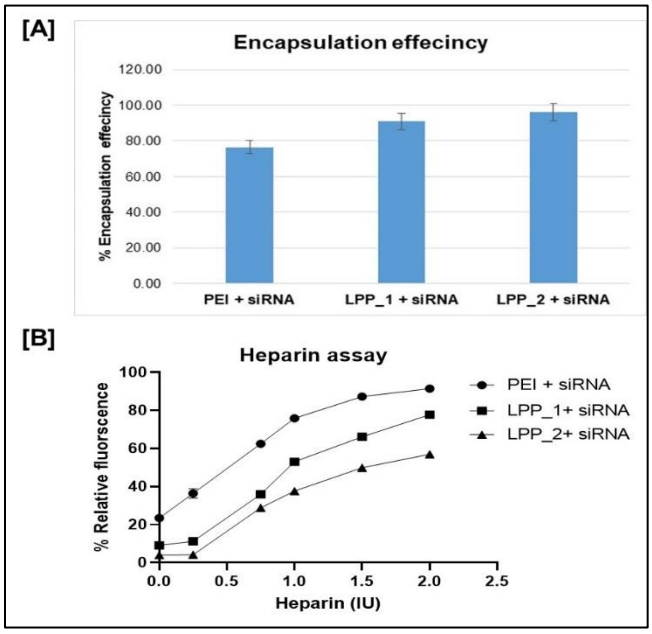


Figure 8. A. Encapsulation efficiency of siRNA in polyplex and LPPs (LPP_1-siRNA and LPP_2-siRNA) and 2B. siRNA release from all the complexes in the presence of different concentrations of heparin was determined using an ethidium bromide intercalation assay (***$p < 0.0001$). Results are represented as mean ± SD (n = 3).

The study showed that lipopolyplexes (LPPs) remained stable even in the presence of heparin, a polyanion. LPPs maintained the integrity of nucleic acids when exposed to heparin doses of up to 2 IU, while non-lipidated polyplexes became unstable at 0.25 IU heparin. Lipidation of the polyplexes in LPPs improved their stability against heparin and other anions, protecting the siRNA payload from degradation by serum components, RNases, and heparin.

These LPPs were then analyzed for their in-vitro toxicity and transfection efficiency *in-vitro* as well as *in-vivo*

ii. Study of siRNA and Chitosan interactions

Medium molecular weight Chitosan was purchased from Sigma Merck, India. Scrambled siRNA was procured from Sigma Merck, India. TPP

a. Formation of nanoparticles of polymer (chitosan)

Method: Chitosan (CS) nanoparticles were created using the ionic gelation method with tri-poly phosphate (TPP). CS was dissolved in glacial acetic acid and adjusted to pH 5.5 with sodium acetate buffer. TPP solution was added to CS under stirring, forming nanoparticles. Different CS: TPP ratios were used to achieve desired particle size. The nanoparticles were then incubated with siRNA. The optimized CS-TPP nanoparticles were analyzed for their ability to form complexes with siRNA using a gel retardation assay.

Results: Chitosan, a biodegradable polymer with a positive charge, is commonly used for siRNA delivery due to its ability to condense siRNA. Medium molecular weight chitosan is preferred due to its lower *in-vivo* toxicity. However, it may have slightly lower transfection efficiency. The initial concentration of chitosan affects the size and viscosity of the CS-TPP nanoparticles during preparation.

Table 1. Optimizes batches of Chitosan nanoparticles

Batch code	CS: TPP	Particle size	PDI	Zeta potential (mV)
CS_2	2:1	195.1±10.12	0.141±0.01	+27.0
CS_4	2:1	207.0±14.68	0.237±0.08	+21.3

b. Study of polymer (chitosan) with scrambled siRNA

Method: To check the condensing ability of optimized CS-TPP nanoparticles to siRNA, a gel retardation assay was performed. The optimized nanoparticles were centrifuged at 8000 rpm for 15 min in eppendorf® centrifuge 5810 R and the nanoparticle pellet was dispersed in HEPES buffer pH 7.4. Different w/w ratios of CS-TPP nanoparticles and siRNA were incubated at 37°C for 30 min and analyzed on 2.5 % w/v agarose gel using gel electrophoresis at 60 V for 45 min.

Results: Batch CS_4 nanoparticles were centrifuged and suspended into HEPES buffer pH-7.4 scrambled siRNA also dissolved in the same buffer.

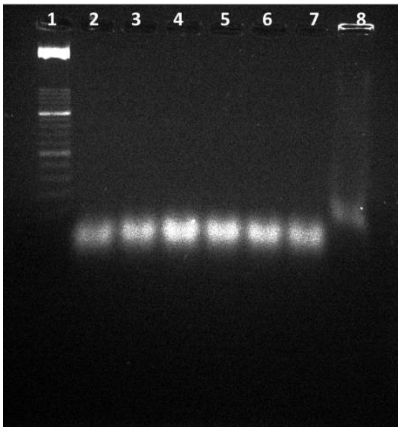


Figure 9. Gel retardation of CS-TPP nanoparticles and siRNA(Lane sequence- Lane1- 50 bp DNA ladder, Lane 2- Plain siRNA (0.33 ug), Lane 3-0.5 ug CS-TPP, Lane 4-1.25 ug CS-TPP, Lane 5- 2.5 ug CS-TPP, Lane 6- 3.75 ug CS-TPP, Lane 7- 4.5 ug CS-TPP, Lane 8- 196 ug CS-TPP.

We found that a maximum quantity of CS-TPP nanoparticles was required for complete condensation of siRNA which might lead to toxicity. So we decided to explore the further modification of CS to increase its complexation and transfection with siRNA

c. Modification of Cs

Cs were modified with arginine and analyzed for their complexation ability. Further, the chitosan-arginine-siRNA was lipidated with anionic liposomes to form LPP. These LPPs were then analyzed further for their stability and transfection efficiency.

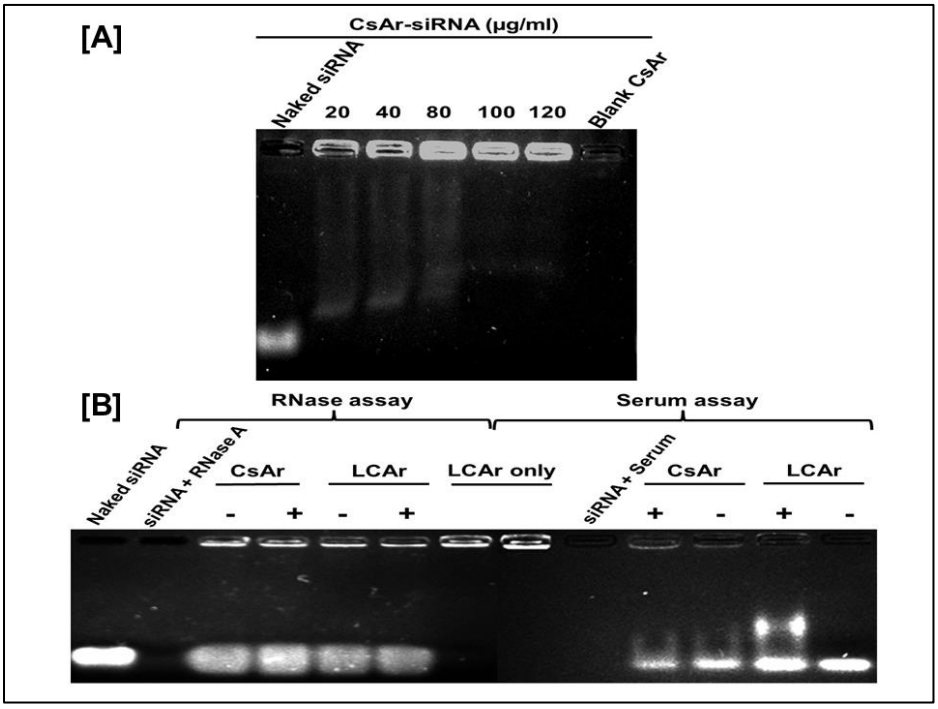


Figure 10. Gel retardation assay. A. Binding efficiency of siRNA and CsAr complexes determined by 2.7%w/v agarose gel electrophoresis. Different concentrations of CsAr (20-120 ug/ml) were added into the fixed concentration of siRNA (20 pmol_ and compared with naked siRNA band intensity (uncomplexed siRNA-20pmol)

B. RNase and serum stability of CsAr and LCAr complexes. The + denotes the sample with serum/RNase treatment and the – denoted the sample without serum/RNase treatment.

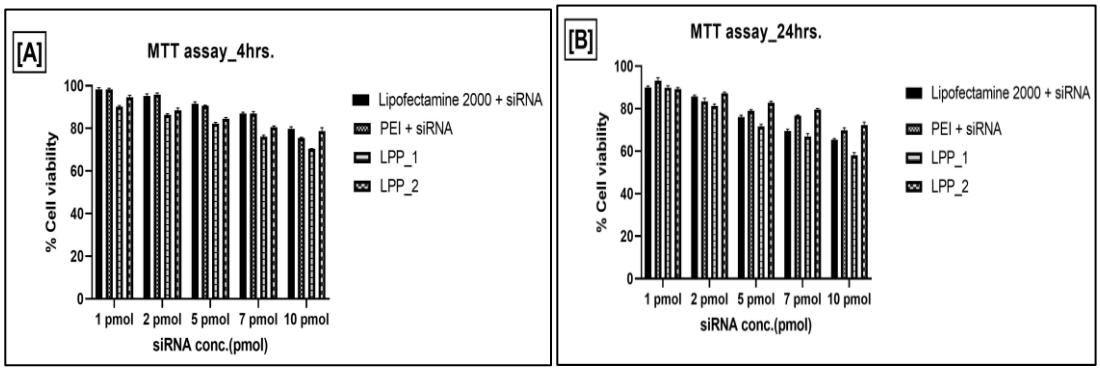
There is no degradation of siRNA from the formulations in the presence of RNAase. The bands with treatment and without treatment were intact. These complexes were further analyzed for their cytotoxicity on retinal cells.

5.6.Toxicity studies

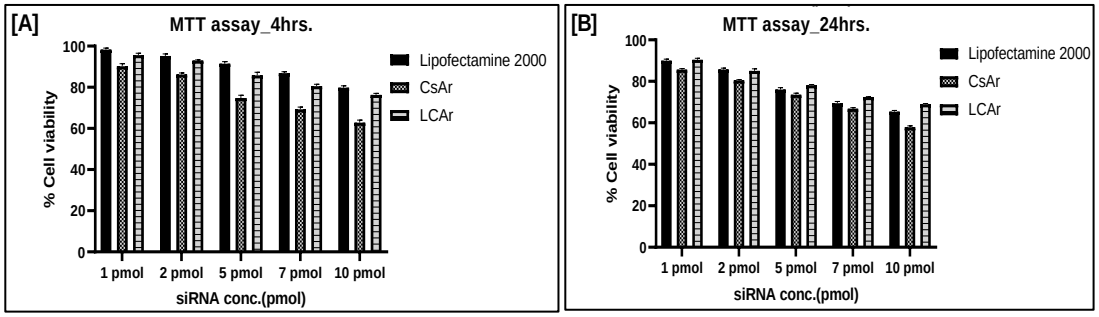
i. MTT assay

Method: The cytotoxicity of siRNA complexes was determined using MTT assays. ARPE19 cells were seeded onto 96-well plates at a density of 5000 cells/well. Cells were exposed to different concentrations of siRNA-containing complexes. After 4 and 24 h incubation, cell media were replaced with 200 μL of 0.25 mg/mL MTT solution added to each well. After incubating for 4 h with MTT solution, the culture medium was removed and 100 μL of dimethyl sulfoxide (DMSO) was added. Reduction in cell viability was measured by colorimetry at 570 nm wavelength using an enzyme-linked immunosorbent assay 253 (ELISA) plate reader (Bio-Rad, USA). Cell viability of each group was expressed as a relative percentage to that of negative control. Cells treated with PBS (phosphate buffer saline) were considered as negative control and commercially available non-viral lipid transfecting carrier Lipofectamine2000 (Invitrogen, USA) was used as positive control[29].

Results: All formulations showed cell viability above 50% at the highest concentration used in the assay i.e. 10pmol of siRNA. Hence, all formulations are safe and can be used for in-vitro transfection assessment.



(a)**siRNA-PEI complexes**



(b) **CsAr-siRNA complexes**

Figure 11 (a)&(b). Cytotoxicity of formulations complexed with increasing doses of siRNA in ARPE19 cells. Cells were treated with siRNA complexes or Lipofectamine2000 and incubated for 4 and 24 h and then viability was measured using MTT assay (n= 5).

ii. Haemolysis assay:

Method: Blood samples (0.8ml/rat) were collected from the Sprague Dawley Rats by retro-orbital puncture and pooled (Animal care was observed as per the guidelines of CPCSEA — Committee for the Purpose of Control and Supervision of Experiments on Animals, India). All blood samples were heparinised for further use. The blood samples were centrifuged at 3000 rpm and washed thrice with normal saline to remove plasma components. The cell suspension was diluted to 10^8 cells/ml. This suspension of volume 100 ul was added to different

concentrations of siRNA complexes. Haemolysis potential of different siRNA complexes in the formulations was determined over siRNA doses upto 10 pmol. Triton-X 100 (0.5% v/v) and normal saline were used as positive and negative control respectively. Treated erythrocyte dispersions were incubated at 37 °C for 4hrs[30]. After incubation, samples were centrifuged at 3000 rpm for 5min and supernatants were analyzed for UV absorbance at 541 nm wavelength against normal saline. The percentage of haemolysis was determined using the following expression:

% haemolysis = (A sample/A positive control) * 100

Results:

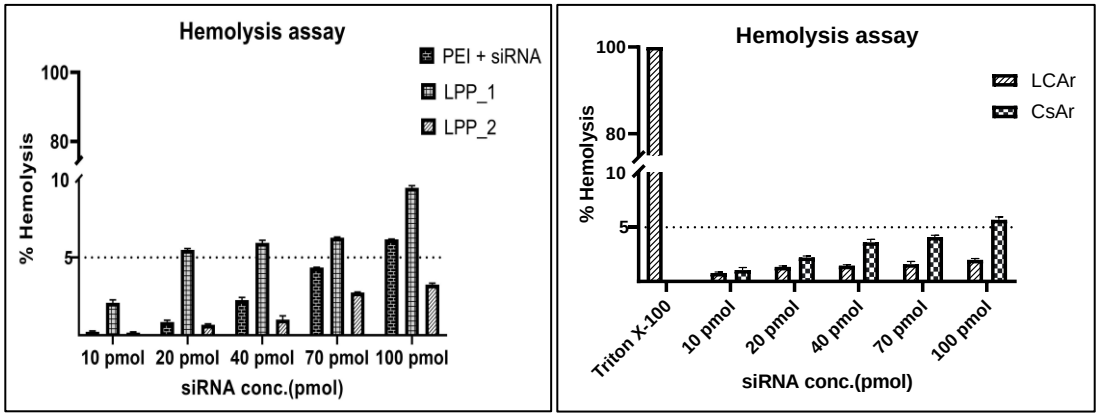


Figure 12(a)&(b). Hemolysis study of siRNA complex formulations at different doses of siRNA was performed.

A hemolysis study was carried out to evaluate the hemolytic potential of prepared complexes. The hemolysis observed with different formulations as compared to that with Triton-X 100 is shown graphically in Fig.12 All liposomal formulations showed less than 4% hemolysis.

5.7.Transfection of ARPE-19 with optimized formulations

Method: siRNA_2 loaded in the complexes was evaluated for their efficiency in downregulating target gene mRNA. Studies were carried out using the commercial transfection agent Lipofectamine®2000 as positive control and plain cells as negative control. Three concentrations of siRNA i.e. 50pmol, 100pmol, and 150pmol have been selected and transfections were carried out in a 6-well plate according to the manufacturer's instructions. The transfections were carried out in triplicate. After four hours, the serum-free medium was replaced with Dulbecco's Modified Essential Medium complete media (without antibiotics). After 48 hours, the cells were lysed using TRIzol reagent (Invitrogen), and mRNA was isolated. The isolated mRNA was converted to cDNA using Superscript III reverse transcriptase (Invitrogen). The cDNA was analyzed by PCR using target gene primers to determine whether the mRNA of the target gene was effectively downregulated by siRNA. RT-qPCR was performed using Power SYBR Green Mastermix (Applied Biosystems, Warrington, UK). Reactions were analyzed upon an ABI 7500 real-time PCR machine using the following cycle conditions: 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles at 57°C for 15 seconds, 72°C for 1 minute, and 60°C for 1 minute. Results were normalized against GAPDH expression. The mRNA expression levels were determined according to the $2^{\Delta\Delta Ct}$ methods [26].

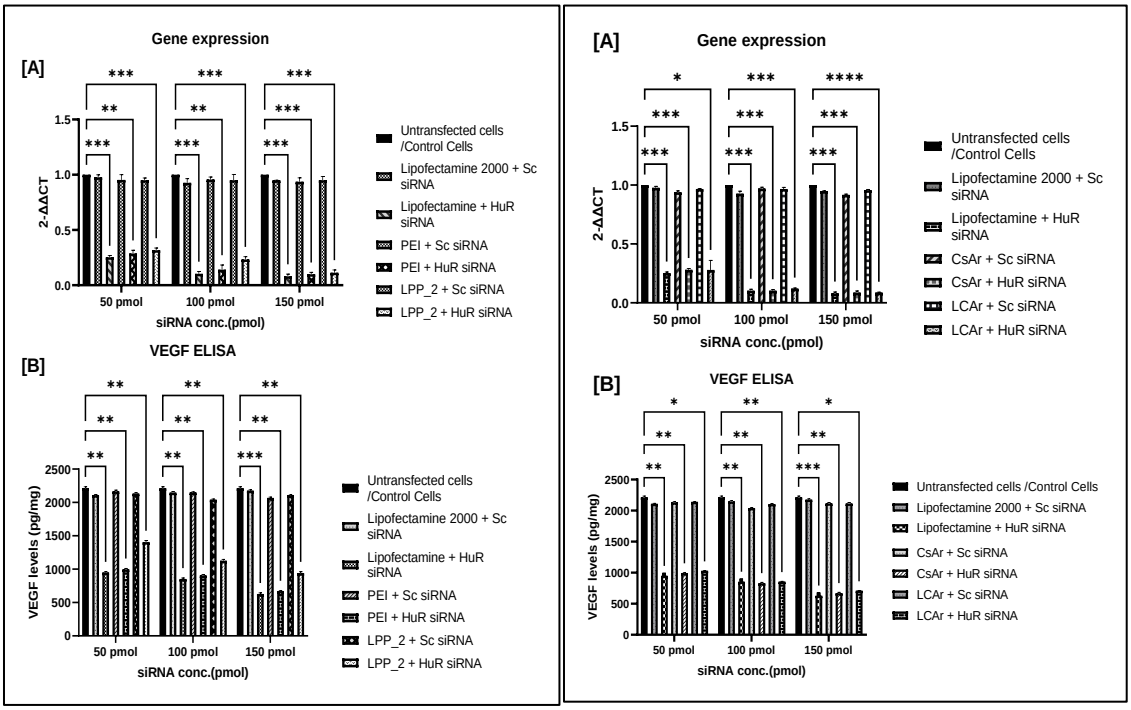


Figure 13. Gene expression study by real-time PCR. This study was performed to find out the effect of different formulations encapsulating designed siRNA on downregulating the target gene. After 48 h, the total reduction in target gene mRNA was determined using RT-PCR by using GAPDH as a housekeeping gene.

Results: As shown in **Figure 13** there is downregulation of target gene mRNA by all the optimized formulations on 48 hrs post-transfection. Dose-dependent downregulation of target mRNA has been observed. VEGF-A protein levels were measured using a Quantikine human VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The results are expressed as VEGF per mg total protein as shown in Figure 13B [31]

5.8.*In-vivo* efficacy study

Method: Male Sprague-Dawley rats were kept in controlled conditions with a 12-hour light/dark cycle, appropriate temperature, and humidity. They were fed standard rat chow

and had access to water ad libitum. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) and conducted following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Diabetes was induced using streptozotocin (STZ) injection, and animals with blood glucose levels above 250 mg/dl were selected for the study. SiRNA and other treatments were administered via intraocular injection. After the study period, the animals were euthanized, and retinal tissue was collected for analysis. Ophthalmoscopy and fundus examination were performed before and after the injections.

Results: It has been observed that all formulations encapsulating rat siRNA when injected in STZ-rats restored target protein to the control level in the retina, as shown in Fig. 14A. and 15A. No significant changes in target protein level occur in control animals treated with siRNA. The gene expression of the target protein has also seen significantly reduced by complexing agent1, CsAr, and LCAr as shown in Figure 14B and 15 B. Similarly, VEGF levels in the retinal tissue were also been reduced significantly by all formulations including Triamcinolone injections as shown in figure 14C and 15C.

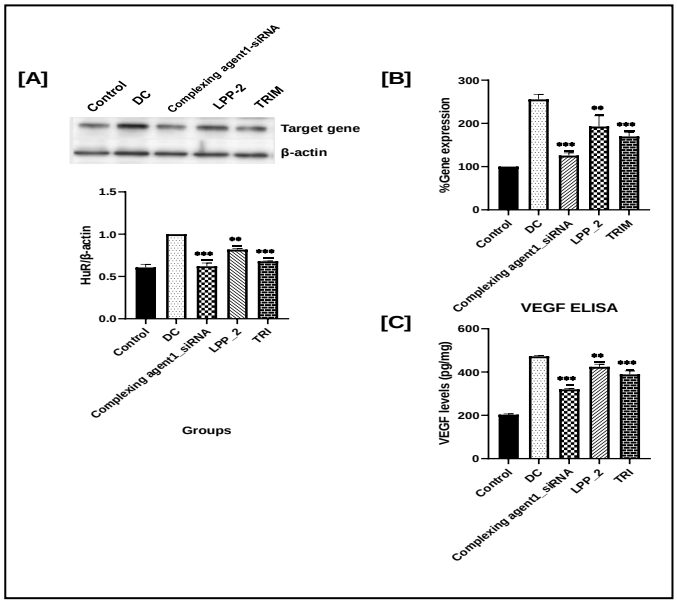


Figure 14. *In-vivo* efficacy study of Complexing agent1 and LPP_2 complexed with rat siRNA. A. Representative images of western blot along with the densitometric evaluation. B. Represents the % Gene expression of the target gene concerning the GAPDH (housekeeping gene) after real-time analysis. C.VEGF levels in retinal tissue expressed as pg/mg of total protein isolated from the rat retinal tissue (n=6).

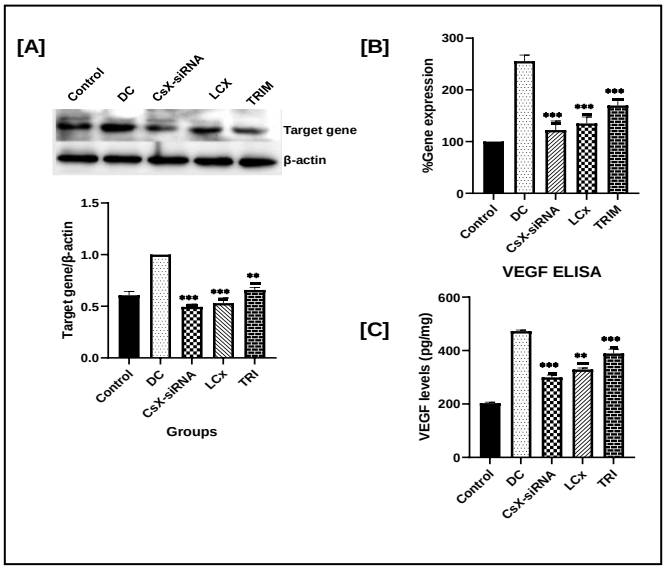


Figure 15. *In-vivo* efficacy study of CsAr and LCAr complexed with rat siRNA. A. Representative images of western blot along with the densitometric evaluation. B. Represents the % Gene expression of the target gene concerning the GAPDH (housekeeping gene) after real-time analysis. C.VEGF levels in retinal tissue expressed as pg/mg of total protein isolated from the rat retinal tissue (n=6).

6. Conclusion

This study aimed to investigate the use of siRNAs to downregulate the expression of VEGF-A by targeting the HuR protein in diabetic retinopathy. In-vitro experiments demonstrated successful downregulation of HuR mRNA, leading to reduced levels of HuR and VEGF-A proteins. Lipopolyplexes (LPPs) were formulated using a combination of polyethyleneimine and liposomes for efficient delivery of HuR-siRNA. LPPs showed stability and enhanced cellular uptake. In-vivo experiments in rats with diabetic retinopathy further confirmed the effectiveness of LPPs in reducing HuR and VEGF levels. This approach holds promise for treating diabetic retinopathy.

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